IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Marc Pignot et al.
Application No.	:	09/744,641
Filed	:	January 26, 2001
For	:	NEW COFACTORS FOR METHYLTRANSFERASES

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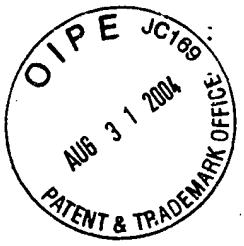


Examiner	:	Josephine Young
Art Unit	:	1645
Docket No.	:	630182.401USPC
Date	:	July 23, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

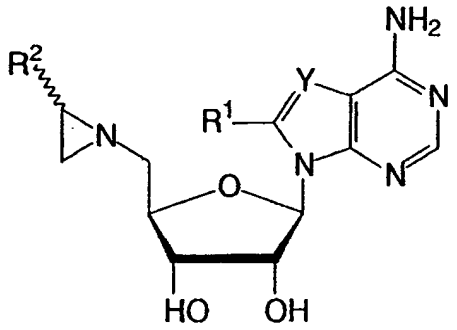
1. I, Elmar Weinhold, am a co-inventor with Marc Pignot, on the above-identified patent application.

2. I am an expert in the field of synthetic chemistry and was an expert at the time of the invention. At the time of the invention I was employed as a group leader at Max-Planck-Gesellschaft zur Foerderung der Wissenschaften, assignee of the above-referenced patent application. Presently I am a Professor of Organic Chemistry at the RWTH Aachen (Rheinisch-Westfälische Technische Hochschule; Technical University of Aachen). My resume is attached as documentation of my credentials.

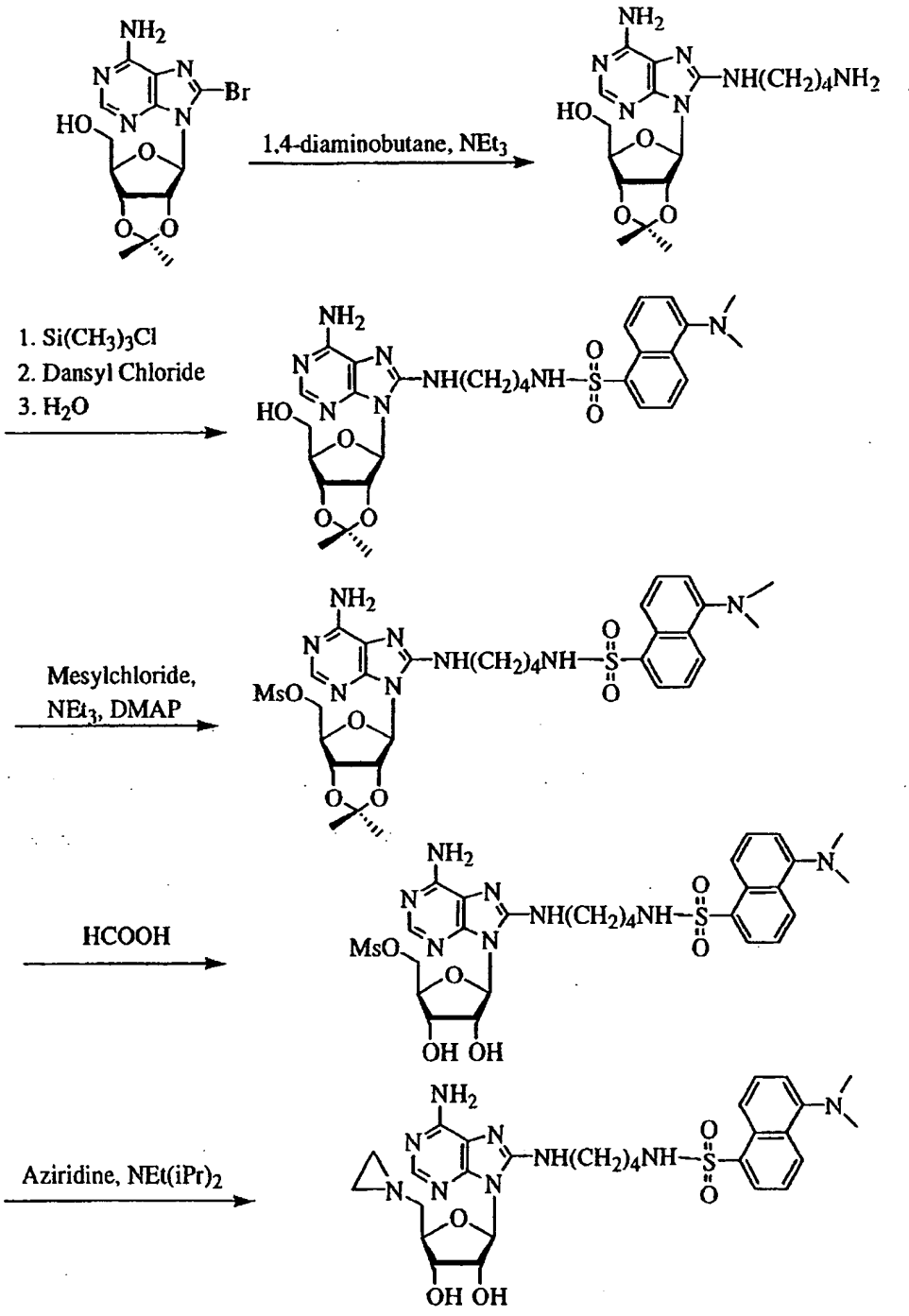
3. I declare that one skilled in the art at the time of the invention using the teaching of the specification, including the exemplary protocols as set forth in Examples 1 and 2, pages 19 to 30 of specification, and variations thereof, and other protocols known in the art at the time of the invention, could have successfully made and used the claimed compounds using only routine screening of alternatives.

As set forth in the specification, compounds of formula (I):

(I)



can be prepared by the following exemplary Reaction Scheme, in which Y is N, R^1 is $-NH(CH_2)_4NHR^4$, R^4 is dansyl, and R^2 is hydrogen (see, *also,* Reaction Scheme 6 on page 14 of the specification):

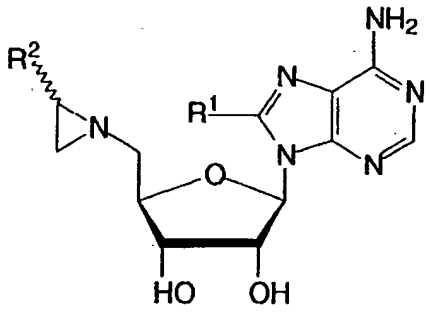


In particular, reaction of 8-bromo-2',3'-O-isopropylidene adenosine with 1,4-diaminobutane yields the protected adenosine derivative with an aminolinker at the 8 position (see, *e.g.*, Compound 1.1 of Example 2 in the specification). Transient protection of the 5'-hydroxy group with $Si(CH_3)_3Cl$, coupling of dansyl chloride with the primary amine of the aminolinker, and removal of the 5' hydroxyl protecting group leads

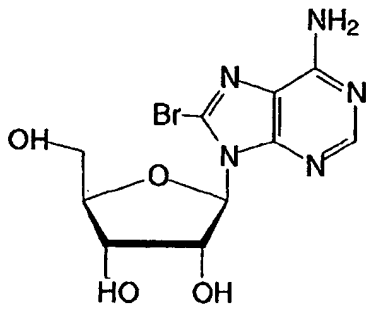
to the protected adenosine derivative with a fluorescent marker on the 8 position (see, *e.g.*, Compound 1.2 of Example 2 in the specification). This intermediate is then reacted with mesylchloride to yield the mesylate derivative (see, *e.g.*, Compound 1.3 of Example 2 in the specification). Removal of the isopropylidene protecting group under acidic conditions followed by reaction with aziridine affords a cofactor of formula (I).

In a similar manner, other compounds of formula (I) wherein Y is N, as represented below as formula (Ia), can be readily synthesized from a compound of formula (IIa) as set forth below according to the teaching of Reaction Scheme 6 and Example 2 of the specification as set forth above. The compound of formula (IIa), *i.e.*, 8-bromoadenosine, was commercially available from Aldrich Co. at the time the above-identified patent application was filed.

(Ia)



(IIa)

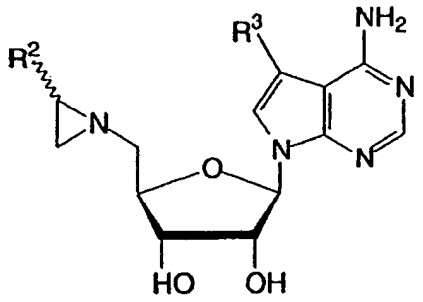


In particular, 8-bromoadenosine can be readily converted to 8-bromo-2',3'-O-isopropylidene adenosine under procedures well known to one of ordinary skill in the organic chemistry field. The bromo substituent at the 8-position can then be replaced by a diamine, such as $NH_2(CH_2)_nNH_2$ (where n is 1-3 or 4-250) or $NH_2(C_2H_5O)_nC_2H_5NH_2$ (where n is 1-250), under known amination conditions to form the appropriate intermediate corresponding to the intermediate prepared from 1,4-

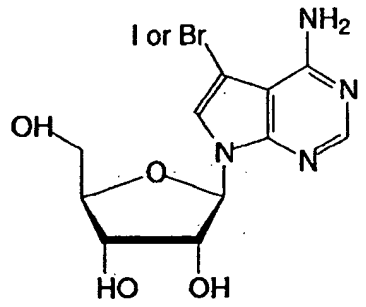
diaminobutane in Reaction Scheme 6. Such diamines would be considered by one skilled in the art to be homologues of $NH_2(CH_2)_4NH_2$ (1,4-diaminobutane) and, as such, would be expected to have comparable physiochemical properties to 1,4-diaminobutane in preparing the desired intermediates. The intermediates so prepared may then be treated with a compound of the formulae $XC(O)R^{4a}$ or $XS(O)_2R^{4a}$ (where X is bromo or chloro and R^{4a} is the rest of the R^4 group) under standard acylation or sulfonylation conditions to prepared compounds of the invention where R^4 is attached to an aminolinker. R^4 is defined by the specification as being common modifiers for biological molecules and that representative R^4 groups can be fluorophores, affinity tags, crosslinking agents, chromophores, proteins, peptides, amino acids, nucleotides, nucleosides, nucleic acids, carbohydrates, lipids, PEG, transfection reagents, beads and intercalating agents. Most, if not all of the compounds that could be used to afford the R^4 group to the claimed compounds are known to be reactive to free amine groups, and therefore can be easily reacted with the intermediate so formed in order to arrive at a compound of the invention. For example, the compound providing the R^4 group for the compounds of the invention in Reaction Scheme 6 is dansyl chloride, thereby forming a compound of the invention where R^4 is dansyl. Other compounds providing the R^4 group may be similarly reacted under conditions known to one skilled in the art with the intermediate to form compounds of the invention wherein R^1 is $-NH(CH_2)_nNHR^4$, or $-NH(C_2H_5O)_nC_2H_5NHR^4$, and R^4 is other than dansyl. The 5'-OH of the ribose moiety of the intermediate so formed can be activated with a good leaving group such as MsCl, as illustrated in Reaction Scheme 6, to form the corresponding –OMs group. Following deprotection of the hydroxyl groups of 2' and 3' position, 5'-OMs can then be replaced with aziridine to form a compound of formula (I).

Likewise, compounds of formula (I) when Y is $-CR^3$, as represented below as Formula (Ib), can be readily synthesized from a compound of formula (IIb) as set forth below. Compound of formula (IIb), *i.e.*, 7-iodo(or bromo)-7-deaza-adenosine, also called 5-iodotubercidin, is commercially available through Sigma-Aldrich.

(Ib)

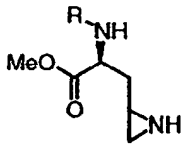


(IIb)



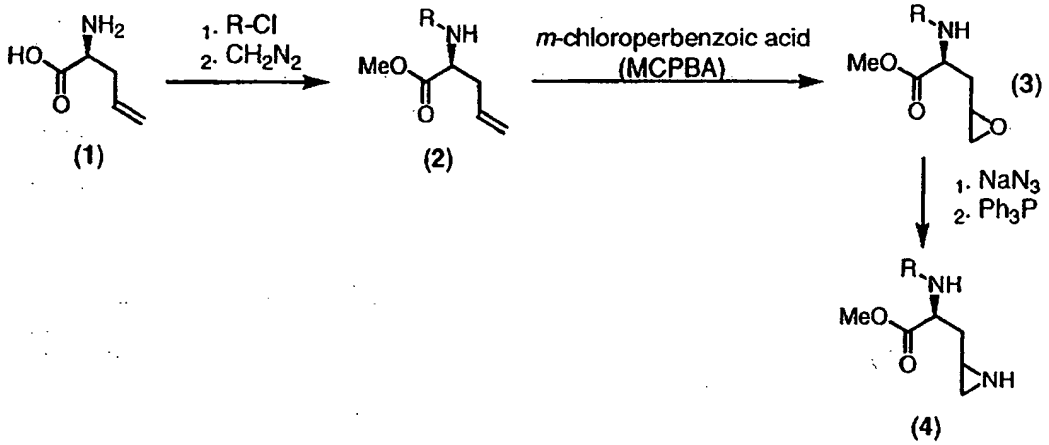
The halogen substitute (Br or I) at the 7 position of the compound of formula (IIb) is readily replaceable by diamines such as $NH_2(CH_2)_nNH_2$ and $NH_2(C_2H_5O)_nC_2H_5NH_2$ in a manner similar to that described above for compounds of formula (IIa), wherein Br at the 8 position is replaced with such a diamine. Consequently, compounds of formula (Ib) where R^3 is $-NH(CH_2)_nNHR^4$ or $-NH(C_2H_5O)_nC_2H_5NHR^4$, can be readily prepared by one skilled in the art according to the teaching of the specification and procedures and reagents known at the time the above-identified patent application was filed.

Furthermore, as one skilled in the art can readily appreciate, a compound of Formula (I) having a substituent on the aziridine ring can be synthesized according to Reaction Scheme 6 of the specification by replacing aziridine with an appropriately substituted aziridine. For example, when R^2 is $-CH_2CH(COOH)NH_2$, a suitably substituted aziridine precursor can be compound (4) as shown below:



(4)

Compound (4) can be readily prepared from a corresponding epoxide according a synthetic methodology known to one skilled in the art at the time of the invention. As exemplified by the following reaction scheme, L-allyl glycine (1, commercially available from Fluka) can be first protected to afford compound 2 in an art known manner. The double bond in 2 is then oxidized to provide an epoxide (3) in the presence of m-chloroperbenzoic acid, a well-known oxidizing agent. The expoxide (3) is then converted to the substituted aziridine (4) under a known reaction condition involving NaN_3 and Ph_3Ph (see, *e.g.*, Legters J. *et al.*, Tetrahedron Lett. **1989**, 30, 4881-4884).



Once compound (4) is attached to the adenosine component of Formula (I) according to the last step in Reaction Scheme 6, the aziridine component can be deprotected following art-known methods to provide a compound of Formula (I) wherein R^2 is $-CH_2CH(COOH)NH_2$.

4. I further declare that one skilled in the art could have used routine protocols known in the art at the time of the invention, including those described in the instant specification, to determine if any of the compounds of the invention acts as a co-factor for a S-Adenosyl-L-methionine (SAM) dependent methyltransferase.

In particular, Examples 1.3 and 2.2 of the specification provides detailed descriptions for preparing the enzymes M·TaqI and M·HhaI, and conducting enzymatic reactions, all of which are routine laboratory procedures known to one skilled in the art. Accordingly, a

screening protocol having general applicability based on these examples can be carried out in the following manner: The enzyme-catalyzed reaction can be carried out in a mixture (500 µl) of cofactor-free methyltransferase (5 nmol, 10 µM), a suitable substrate to the particular methyltransferase (5 nmol, 10 µM), a compound of formula (I) (10 nmol, 20 µM), Tris acetate (20 mM, pH 6.0), potassium acetate (50 mM), magnesium acetate (10 mM) and Triton X-100 (0,01 %) at 37°C. The progress of the reaction can be monitored by anion exchange chromatography (Poros 10 HQ, 10 µm, 4,6 x 10 mm, PerSeptive Biosystems, Germany). The product (which is the result of the enzyme-catalyzed transfer of the compound of formula (I) to the substrate) can then be eluted with aqueous potassium chloride (0.2 M for 5 min, followed by a linear gradient to 0.5 M in 5 min and to 1 M in 30 min) in Tris hydrochloride buffer (10 mM, pH 7.0).

Additionally, the specification, by way of detailed examples, provides three alternative means to analyze the product resulting from the transfer of a compound of formula (I) to a substrate in the presence of a suitable methyltransferase.

First, according to Example 1.3.1 on page 20 of the specification, the product can be analyzed directly by reversed-phase HPLC-coupled electrospray ionization mass spectrometry. More specifically, RP-HPLC/ESI-MS can be performed with an ion-trap mass spectrometer (LCQ, Finnigan MAT, Germany) equipped with a micro HPLC system (M480 and M300, Gynkotek, Germany). The product can be purified by anion exchange chromatography, followed by desalting by repeated addition of water and ultrafilteration (Microsep 3K, Pall Filtron, Northborough, MA, USA). The product solution can then be injected onto a suitable capillary column (for example, Hypersil-ODS, 3 mm, 150 x 0.3 mm, LC Packings, Amsterdam, Netherlands) and eluted with a linear gradient of acetonitrile (7-10% in 10 min, followed by 10-70% in 30 min, 150 µl/min) in triethylammonium acetate buffer (0.1 M, pH 7.0). The molecular weight obtained can be compared to the calculated molecular weight of the product.

Second, the product can be analyzed by electrospray ionization mass spectrometry using direct infusion according to Example 1.3.1 on page 21 of the specification. More specifically, a double focusing sector field mass spectrometer MAT 90 (Finnigan MAT, Germany) equipped with an ESI II electrospray ion source in the negative ion mode can be used. The desalted product in a aqueous solution and a liquid sheath flow (2-propanol) can then be delivered using a Harvard syringe pump (Harvard Apparatus, USA). The molecular weight of the product obtained from the electrospray mass spectra can then be compared to its calculated molecular weight.

Third, for a product wherein the substrate is an oligodeoxynucleotide (or oligonucleotide), the product can be analyzed by mass spectroscopy following an initial step of enzymatic fragmentation, according to Example 1.3.1 on page 21 and Example 2.2 on page 26. Specifically, a purified product of transferring a compound of formula (I) to an oligodeoxynucleotide (or oligonucleotide) can be dissolved in potassium phosphate buffer (10 mM, pH 7.0, 228 µl) containing magnesium chloride (10 mM), DNase I (2.7 U), phosphodiesterase from Crotalus durissus (0.041 U), phosphodiesterase from calf spleen (0.055 U) and alkaline phosphatase (13.7 U) and incubated at 37°C for 20 h. An aliquot (100 ml) can be injected onto a reversed-phase HPLC column (Hypersil-ODS, 5 mm, 120 Å, 250 x 4.6 mm, Bischoff, Leonberg, Germany), and the products can be eluted with a gradient of acetonitrile (0-10.5% in 30 min followed by 10.5-28% in 10 min and 28-70% in 15 min, 1 ml/min) in triethylammonium acetate buffer (0.1 M, pH 7.0). Beside the deoxynucleosides dC, dA, dG, T, and dA^{Me}, a new compound eluted can be found. The new compound can be isolated and detected by ESI-MS (LCQ connected to a nanoelectrospray ion source, Finnigan MAT, Germany). The observed mass is then compared with the calculated molecular mass of the product that is expected to be obtained by transferring a compound of formula (I) to the oligodeoxynucleotide substrate.

5. I declare that one skilled in the art could have used routine protocols known in the art at the time of the invention, including those described in the instant specification, to

determine if a putative methyltransferase could have complexed with an aziridine derivative of the present invention.

It was known in the art at the time of the invention that SAM-dependent methyltransferases for diverse substrates such as DNA, RNA, protein, peptide and even small molecules, had common catalytic domain(s) for binding the SAM cofactor. As demonstrated above, the instant invention is directed to the discovery that compounds of formula (I) behave in substantially the same manner as SAM in the presence of two particular DNA methyltransferase, M·TaqI and M·HhaI. See, for example, Reaction Scheme 7 of the specification. This result indicates to one skilled in the art that the compounds of the invention occupy the same catalytic domain of the DNA methyltransferases as SAM does, and would therefore function in the same manner for other SAM dependent methyltransferases due to the common catalytic domains of such enzymes.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted

Date: July 23, 2004

Elmar Weinhold

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Date of Birth: July 13, 1960

Place of Birth: Berlin, Germany

Positions/Education:

since 06.00	**Professor of Organic Chemistry** at the Institut für Organische Chemie der RWTH Aachen, Germany.
07.93 – 05.00	**Group leader** at the Department of Physical Biochemistry (*Prof. Roger Goody*), Max-Planck-Institut für molekulare Physiologie, Dortmund, Germany. **Habilitation in bioorganic chemistry** at the Fachbereich Chemie, Universität Dortmund, Germany, with the title: Synthesis of modified duplex oligodeoxynucleotides and cofactor analogues for structure-function studies of DNA methyltransferases.
03.93 – 05.93	**Visiting scientist** at the New England Biolabs, Inc., Beverly, MA, USA.

03.91 – 02.93	**Postdoc** at the Department of Chemistry, Harvard University, Cambridge, MA, USA. Postdoctoral fellow of the Deutsche Forschungsgemeinschaft in the laboratory of *Prof. Jeremy R. Knowles* with the project title: Binding studies of synthestic sialic acid derivatives and influenza A hemagglutinin
07.86 – 02.91	**Ph.D.** at the Laboratorium für Organische Chemie der ETH-Zurich, Switzerland. Ph.D. thesis in the group of *Prof. Steven Benner* with the title: Protein engineering: A method for understanding the relationship between structure and activity of alcohol dehydrogenase from yeast.
02.86 – 06.86	**Graduate Student** at the Department of Chemistry, Harvard University, Cambridge, MA, USA.
03.80 – 01.86	**Diploma in Chemistry** (very good) at the Freie Universität Berlin, Germany. Diploma thesis with *Prof. Johann Mulzer* in the field of asymmetric synthesis.

Peer-reviewed publications

26. A. David, N. Bleimling, C. Beuck, J.-M. Lehn, E. Weinhold, M.-P. Teulade-Fichou, "DNA mismatch-specific base flipping by a bisacridine macrocycle", *ChemBioChem*, submitted.
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